Mass transport controlled oxygen reduction at anthraquinone modified 3D-CNT electrodes with immobilized Trametes hirsuta laccase

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Carbon nanotubes covalently modified with anthraquinone were used as an electrode for the immobilization of Trametes hirsuta laccase. The adsorbed laccase is capable of oxygen reduction at a mass transport controlled rate (up to 3.5 mA cm⁻²) in the absence of a soluble mediator. The storage and operational stability of the electrode are excellent.

Introduction

Enzymatic biofuel cells are extensively investigated as an alternative to fuel cells relying on precious metal catalysts.1,2 In particular, physiological operating conditions are targeted for implantable biofuel cells for medical applications.3,4 There are a number of key demands on electrodes comprising the biofuel cell. The bioelectrodes have to have suitable potential and pH optima as well as resistance to chemical components such as Cl⁻ ions. High current densities are desirable and these should be achieved without a soluble mediator, i.e. the mediator has to be co-immobilized with the enzyme or the redox enzyme has to be in direct electrical contact with the electrode matrix. It is also essential for the enzyme to be immobilized in a stable fashion and not to deactivate during continuous operation.

Fungal, high potential laccases have been extensively used as the cathodic bioelectrodes catalysing oxygen reduction to water. They were shown to be capable of direct electron transfer, particularly when bound to electrodes made of carbonaceous materials.5–14 This electronic communication was found to be efficient way of achieving this and was employed previously to construct laccase based biocathodes.19–25 This communication presents results for a biocathode based on direct electrochemistry of immobilized Trametes hirsuta laccase (ThL). The laccase was immobilized on hierarchically grown carbon nanotubes which were modified by anthraquinone groups. This particular functionalization was shown in earlier work to improve enzyme retention on the electrode and facilitate direct electron transfer to the enzyme active centre.17 Modified electrodes were characterized by cyclic voltammetry and rotating disk electrode studies. Laccase catalysed oxygen reduction was found to be mass transport controlled over the investigated mass transfer coefficient range. The combination of the hierarchically structured carbon nanotube electrode matrix and efficient enzyme wiring also resulted in very high storage and operational stability.

Experimental

Materials and instrumentation

Tetrabutylammonium tetrafluoroborate (TBATFB) (electrochemical grade, Fluka), N-Boc-ethylenediamine (Sigma-Aldrich), citric acid (BDH), trisodium citrate (Sigma-Aldrich), 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (Sigma), N,N-diisopropylethylamine (DIEA) (Sigma-Aldrich), acetonitrile (HPLC grade, Rathburn, UK), N,N-dimethylformamide (DMF) (synthesis grade, Fisher Scientific), 4 M HCl in dioxane (Fluka), ethanol (EtOH) (analytical grade, Fisher Scientific) and O-(benzotriazol-1-y1)-N,N',N,N'-tetramethyluronium hexafluorophosphate (HBTU) (Novabiochem) were used as received. All aqueous solutions were prepared with reagent grade water from a Purite purification system. Argon and oxygen (BOC) were used to purge the solutions. ThL was a generous gift from VTT Technology (Finland). When not used the enzymes were stored in citrate or acetate buffer, frozen at −18 °C. Electrochemical measurements were performed in glass, thermostated (25.0 ± 0.5 °C) cells in a standard three electrode arrangement using a μAutoLab type III potentiostat running GPES software (EcoChemie, Netherlands). An AFCPRB rotator (Pine Research Instrumentation, USA) was used for all Rotating
Disk Electrode (RDE) experiments. A saturated calomel electrode (SCE) or saturated mercury/mercurous sulphate electrode (SMSE) were used as reference electrodes, and platinum gauze served as the counter electrode. In the remainder of this article all potentials are quoted with respect to SCE. Current densities were calculated using the geometric area of the underlying graphite disc (0.071 cm²).

**Preparation of 3D-CNT electrodes**

The electrodes were prepared as previously described.²³–²⁵ Electrochemical deposition of iron as a catalyst for the chemical vapour deposition (CVD) growth of carbon microfibers (CMF) and carbon nanotubes (CNT) was carried out using a CHI1030 workstation (CH Instrument, USA) in a solution containing 500 mM FeSO₄·7H₂O and 580 mM MgSO₄·7H₂O. A spectrographic graphite rod (Schunk Kohlenstofftechnik, Germany, 3.05 mm) was used as the working electrode. A multi-potentials pulse sequence (0 mV for 10 s, +600 mV for 2 s) was applied for the electrodeposition of Fe on the bare graphite rod or on the CMF-modified surface. The graphite rods or CMF-modified graphite rods decorated with Fe particles were washed with water and then placed in a quartz holder. The reactor was a horizontal hot-wall quartz tube with a length and an inner diameter of 100 cm and 3 cm, respectively. The quartz tube was inserted into a triple-zone furnace (Carbolite, Germany) and connected to a computer-controlled gas mixing unit. The gas flow rates were adjusted by mass-flow controllers. Gases were supplied by Air Liquide, Germany (purity: H₂ > 99.9999%, He > 99.9999%). For growing CMF, the furnace was first heated to 850 °C using a heating ramp of 20 K min⁻¹ and then to 1150 °C with a heating ramp of 3 K min⁻¹ in a H₂ and CH₄ mixture with a flow rate of 70 mL min⁻¹ and 30 mL min⁻¹, respectively. The temperature was maintained at 1150 °C for 20 min for growing the CMF. After this, the furnace was cooled down to 950 °C under a flow of 100 mL min⁻¹ H₂ and finally to room temperature under a flow of 148 mL min⁻¹ He. For the growth of CNT, the furnace was first heated to 700 °C with a heating ramp of 10 K min⁻¹ and then kept at 700 °C for 30 min in a H₂–He mixture with a flow rate of 100 mL min⁻¹ for each gas. Subsequently, the furnace was heated to 760 °C with a heating ramp of 10 K min⁻¹ and then kept at 760 °C for 30 min to grow CNT in a mixture of H₂ (70 mL min⁻¹) and C₂H₄ (30 mL min⁻¹). After this, the furnace was flushed with a flow of 100 mL min⁻¹ H₂ for 30 min at 760 °C. Finally the furnace was cooled down to room temperature under a flow of 140 mL min⁻¹ He. The CNT modified graphite rods (3 mm diameter) were fitted in Teflon RDE tips (type AFE4TQ050, Pine Research Instrumentation, USA) with a home-made PTFE adaptor ring.

**Modification of 3D-CNT electrodes**

The functionalization with ethylenediamine, followed by coupling of anthraquinone-2-carboxylic acid and enzyme adsorption were achieved as described earlier¹⁷ and are schematically depicted in Scheme 1. The presence of anthraquinone on the electrode surface was verified by cyclic voltammetry in pH 7 phosphate buffer and the coverage estimated from the charge under the voltammetric peaks. After washing with water and drying in a stream of argon 10 µL of laccase solution (3.9 mg mL⁻¹ in 20 mM, pH 5 citrate buffer) was pipetted onto the surface and left overnight at 4 °C. Loosely bound enzyme was removed by extensive washing in pH 5, 0.1 M citrate buffer.

**Results and discussion**

The hierarchically structured CNT–CMF–graphite electrodes were obtained by a sequence of electrochemical deposition of Fe as a catalyst for the following growth of first CMF and then CNT using chemical vapour deposition. The CNT–CMF modified electrodes were functionalized with an amine linker which was subsequently used to couple anthraquinone 2-carboxylic acid. The presence of anthraquinone (AQ) was verified by cyclic voltammetry and its surface concentration quantified from the charge under the voltammetric peaks (Fig. 1). The coverage of AQ (Iₐ/Q) was found to be between 110 and 140 nmol cm⁻² (related to the geometric area of the underlying graphite disc) and gave a good indication of the enhancement in the electroactive area. In comparison with analogously functionalized polished glassy carbon surfaces (Iₐ/Q ≈ 0.5 nmol cm⁻²) the CNT modification yields on average a 250 fold increase in the surface area available for linker attachment and functionalization.

Upon modification with AQ the electrodes were incubated with the enzyme solution, washed and tested for the enzyme presence and activity. No further stabilisation of the enzyme layer (e.g. crosslinking, membrane entrapment) was undertaken.
and its binding is believed to rely solely on hydrophobic interactions with the matrix of chemically modified CNT–CMF. The presence of the active laccase was evidenced by pronounced oxygen reduction currents (Fig. 2) normally not observed in this potential window for CNT or CNT-AQ electrodes. The onset of the oxygen reduction wave was observed at potentials exceeding 800 mV (vs. NHE) clearly pointing to the catalysis by high potential blue laccase.26 The addition of the soluble mediator ABTS did not result in increased current and essentially the same voltammogram was recorded. This suggests that a sufficient amount of enzyme is capable of direct electron transfer between the electrode and the T1 copper and the overall kinetics of enzymatic reaction are much faster than the diffusion of dissolved oxygen. The mass transport limited nature of the recorded enzymatic reaction are much faster than the diffusion of dissolved oxygen (dashed line) recorded after seven weeks of storage. 

ν = 1 mV s⁻¹, stationary electrode, T = 25 °C.

The mass transport limited nature of the recorded currents was further confirmed in RDE experiments (Fig. 3). In the range of available mass transfer coefficients k_m (rotation rates up to 2500 rpm corresponding to k_m = 0.016 cm s⁻¹) the oxygen reduction currents increased linearly with the square root of rotation rate and no kinetic limitation was observed. It has to be stressed that such behaviour was critically dependent on the amount of immobilized nanotubes and consequently the amount of enzyme. For surfaces of lower real surface area (e.g. showing AQ coverage of ~20 nmol cm⁻²) the currents were significantly lower and a kinetically controlled response was observed. The oxygen reduction current densities showed less dependence on w as w was increased, reaching only 1 mA cm⁻² at 2500 rpm compared to 3.5 mA cm⁻² observed for the most active electrodes.

Both storage and operational stability of the biocathode were investigated. When not in operation the electrodes were stored in pH 5 citrate buffer at 4 °C. The storage had virtually no effect on the activity of electrodes towards oxygen reduction as can be seen both in voltammetric (dashed and solid curve in Fig. 2) and RDE (black and red symbols in Fig. 3) experiments. Rather than infrequent testing, a continuous operation is of real significance when considering the use of an electrode as part of a biofuel cell. Operational stability was therefore evaluated by holding the potential at −0.1 V vs. SMSE (0.3 V vs. SCE) and monitoring the bioelectrocatalytic current continuously for 7 days (Fig. 4), while the electrode was rotated at 900 rpm in air saturated, pH 5.0, 0.1 M citrate buffer. The biocathode showed remarkable stability with the current densities decreasing only by approximately 21% over 7 days. A more significant decrease occurs in the first 48 h (11%) followed by stabilised response.

Conclusions

A biocathode based on hierarchically grown nanofibres/nanotubes non-covalently modified with laccase was constructed and evaluated. The modification of carbon nanotubes with anthraquinone via an ethylenediamine linker was found to efficiently wire the enzyme to achieve direct electron transfer between laccase and the electrode matrix, providing high CNT/enzyme loading. The cathode was capable of oxygen reduction limited only by mass transport, which is of paramount importance to maximize the power output of a biofuel cell. The combination of the high surface area of carbon nanotubes and their chemical modification has led to remarkable stability resulting from good enzyme retention and mechanical robustness of the 3D-CNT electrodes, which is a step forward towards enzymatic biofuel cells.
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Notes and references